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(54) Title: PREVENTION OF DENTAL CARIES

(57) Abstract

A penta– to decapeptide containing two or more Arg protecting against dental caries is comprised by the amino acid sequence GlyGlyHisProArgProProArgGlyArgProGlnGlyProProGlnGln. Also disclosed is a composition for preventing dental caries comprising an effective amount of the peptide, and a corresponding method of use.

PREVENTION OF DENTAL CARIES

FIELD OF THE INVENTION

5   The present invention relates to a means for preventing
dental caries, a corresponding method, and compositions
incorporating the means.

BACKGROUND OF THE INVENTION

10

Proline-rich proteins (PRPs) comprise about 70% of the
proteins in saliva. They can be divided into acidic, basic,
and glycosylated PRPs encoded by six clustered genes on
chromosome 12p13.2. PRPs are potential determinants of host
15   susceptibility to dental caries. Large multifunctional 150-
residue PRPs (Lamkin M S and Oppenheim F G, Crit Rev Oral
Biol Rev 4 (1993) 251-9) are known to adsorb to
hydroxyapatite surfaces and inhibit calcium phosphate
precipitation, modulate hydroxyapatite crystal formation as
20   well as mediate adhesion of commensal *Actinomyces* and
Streptococcus species to tooth surfaces. In addition, they
bind to and inactivate ingested plant polyphenols (tannins).
The proline-poor N-terminal 30 residue domain confers
hydroxyapatite- and calcium-binding, while the proline-rich
25   middle/C-terminal domain binds bacteria via the ProGln-
terminus and tannins via proline-rich repeats.

The polymorphism of acidic PRPs involves allelic and post-
translational variants; several common allelic (large PRP-1,
30   PRP-2, Db-s, PIF-s and Pa) and post-translational (small
PRP-3, PRP-4, Db-f and PIF-f) variants have been described.
In regard of biological properties the acidic PRP variants
differ somewhat, and large and small acidic PRPs largely,
between each other. The small 106 residue acidic PRPs

resulting from proteolytic cleavage at Arg106-Gly107 display
poor bacterial adhesion activity but high affinity for
hydroxyapatite surfaces. Both large and small acidic PRPs
are secreted from the acinus cells. After secretion, the

5   acidic PRPs are rapidly enriched on tooth surfaces and
degraded as a consequence of bacterial proteolysis. Also,
multiple phosphorylated peptides reminiscent of PRPs have
been identified in saliva and found to possess increased
affinity for hydroxyapatite surfaces. Some studies

10   (Kousvelari E E et al., J Dent Res 59 (1980) 1430-8), though
not others (Mandel I D & Bennick A, J Dent Res 62 (1980)
943-5), have associated this proteolytic activity to
gingivitis and rate of plaque formation.

15   Arg catabolism to ammonia has been suggested to be a
characteristic of streptococcal biotypes with tooth
protective properties (Andersson C et al. Infect Immun 43
(1984) 555-60; Rogers AH, Aust Dent J 35 (1990) 468-71.
Wijeyeweera R L& Kleinberg, Arch Oral Biol 34 (1989) 55-64;

20   ibid. 43-53). Actually, Arg can control the ecological
relationship between S. sanguis and S. mutans (van der
Hoeven J S et al., J Dent Res 63 (1984) 389-92. Rogers A H
et al., Oral Microbiol Immunol 2 (1987) 172-82), and caries-
susceptible and caries-resistant subjects differ in ability

25   to raise pH after an acidification (Abelson D C & Mandel I
D, J Dent Res 60 (1981) 1634-8; Kleinberg I et al., Proc.
"Microbial aspects of dental caries". Sp. Supp. Microbiol.
Abstracts. IRL, Washington, D.C., 1976, 433-464 pp).

30   Arg catabolism is present in S. sanguis, S. gordonii, S.
anginosus and S. mitis, while it is absent in S. oralis and
cariogenic S. mutans and S. sobrinus (Kilian M et al., Int J
Syst Bacteriol 39 (1989) 471-484). Arg-containing peptides,
notably sialin (Coulter W A et al., Biochem Soc Trans 18

(1990) 337-8) may act as a local pH-buffering agents by
bacterial catabolism of Arg to ammonia via the arginine
deaminase pathway (Floderus E et al., APMIS 98 (1990) 1045-
52).

5

Degradation of acidic PRPs releases oligopeptides which are
transported intra-cellularly and metabolized for nutritional
reasons (Kunji E R et al., Antonie Van Leeuwenhoek 70 (1996)
187-221). Gram-negative bacteria colonizing the gingival
10   pocket, such as *Porphyromonas gingivalis*, express a broad
range of proteases in utilizing proteins as a major source
of energy. In contrast, commensal streptococci and
actinomycetes, which dominate on oral mucosal and tooth
surfaces, express multiple glycosidases in utilizing
15   carbohydrates as a major energy source. Nevertheless,
protease and peptidase activities, such as a trypsin-like
serine endoprotease in S. oralis, S-IgA1 proteases in *S.
sanguis* and *S. oralis* and yet uncharacterized proteolytic
activity, are present in *Streptococcus* and *Actinomyces*
20   species. However, little is known about the degradation of
acidic PRPs by these commensal and early colonizing
bacteria.

While a vast amount of knowledge about saliva constitutents
25   and their possible role in processes leading to dental
caries has been gathered, that knowledge up to know resulted
in only few practically applicable propositions of how to
efficiently prevent dental caries.

30   OBJECTS OF THE INVENTION

It is an object of the invention to provide a means for
protecting dental surfaces against caries.

It is another object of the invention to provide a corresponding method.

It is a further object of the invention to provide a
5   composition for protecting dental surfaces against caries.

Still other objects of the invention will become apparent from the following description of the invention and preferred embodiments thereof, as well as from the appended
10   claims.

DESCRIPTION OF THE INVENTION

The present invention is based on the insight that commensal
15   *Actinomyces* and *Streptococcus* species transform acidic PRPs to small-size peptides, such as pentapeptides. These small-size peptides are transformed into ammonia by the action of certain oral bacteria. The ammonia thus formed protects raises the pH at the dental surface and therby protects the
20   surface against caries.

In this transformation Arg-containing oligopeptides are released and catabolized to ammonia at or near the dental surface. Ammonia increases the pH at the dental surface and
25   thereby counteracts caries which is promoted by a low pH in the oral cavity.

The present invention is also based on the insight that the aforementioned transformation gives rise to peptides with
30   terminal proline or proline-glutamine entities having affinity to dental surfaces and thus competitively protecting them.

Degradation of acidic PRPs by *Streptococcus* and *Actinomyces* species comprises the following features:

(i) step-by-step the large 150 residue PRP-1 protein is degraded into intermediate size peptides and finally into

5   smaller oligopeptides and amino acids. Cell-bound endoproteases with broad substrate specificities are involved in the initial stages of this degradation;

(ii) cleavage of acidic PRPs occurs preferentially in the middle/C-terminal proline-rich 96-140 region; the 106

10   residue PRP-3 is relatively resistant to cleavage, PRP-3-like peptides being generated by all strains and a series of fragments in region 96-140. Both endoprotease specificity *per se* and intrinsic properties of acidic PRPs, such as the extended structure of proline-rich stretches, explains this

15   regional preference of cleavage;

(iii) degradation of PRP-1 releases oligopeptides both immediately and after prolonged degradation. The immediate release of an Arg106Gly107Arg108Pro109Gln110 pentapeptide is indicated by the N-terminal 105 residue Pyr1-Pro104Pro105

20   and the C-terminal 40 residue Gly111-Pro149Gln150 peptides initially generated by *S. gordonii* strain SK12.

Mass spectroscopy identified a N-terminal pyroglutamatic acid (Pyr) post-translational modification of acidic PRPs

25   that blocks sequencing by Edman degradation. The peptide structures derived from degradation of the 150 residue PRP-1 by *S. gordonii* strain SK12 indicate:

(i) the instant release of an Arg106Gly107Arg108Pro109Gln110 pentapeptide together with a N-terminal 105 residue Pyr1-

30   Pro104Pro105 and a C-terminal 40 residue Gly111-Pro149Gln150 peptide;

(ii) the generation of multiple middle/C-terminal peptides with ProGln-termini;

(iii) the cleavage at peptide bonds formed at Pro or Gln residues: Pro95-Pro96, Pro104-Pro105, Pro105-Arg106, Gln110-Gly111, Gln131-Gly132, Pro135-Gln136, Gln136-Gly137, and Gln141-Gly142.

5

Degradation of acidic PRPs by *S. gordonii* SK12 initially generates a pentapeptide, Arg106Gly107Arg108Pro109Gln110, containing two of the five Arg residues in PRP-1.

10   The N-terminal Glu1-Pro104Pro105 peptide derived from PRP-1 by S. gordonii SK12 lacks the bacterial adhesion-promoting ProGln-terminus, but contains the hydroxyapatite binding domain. Since small phosphorylated proline-rich peptides and PRP-3 display increased affinity for hydroxyapatite surfaces

15   (Moreno E C et al., J Biol Chem 257 (1982) 2981-9; Madapallimattam G & Bennick A, Biochem J 270 (1990) 297-304; Lamkin M S et al., J Dent Res 75 (1996) 803-8), such N-terminal peptides can affect bacterial adhesion by occupying hydroxyapatite sites or altering the pellicle mosaic of

20   binding structures. Proteolytic cleavage is known to unmask hidden binding structures (Gibbons R J et al., Arch Oral Biol 35 Suppl (1990) 107s-114s), and strains of *Actinomyces* species recognize diverse sites in acidic PRPs and statherin (Hallberg K et al., Oral Microbiol Immunol 13 (1998) 327-

25   336; Li T et al., Infect Immun 67 (1999) 2053-9). Furthermore, acidic PRP-degradation generated multiple middle/C-terminal peptides with adhesion-active ProGln-termini, potentially inhibiting the adhesion of oral pathogens binding to acidic PRPs and statherin, i. e. *S.*

30   *mutans* (Gibbons R J & Hay D I, J Dent Res 68 (1989) 1303-7), *P. gingivalis* (Amano A et al., Infect Immun 62 (1994) 3372-80) and *Fusobacterium nucleatum* (Xie H et al., Oral Microbiol Immunol 6 (1991) 257-63). Commensal and pathogenic species display high and low avidity binding to acidic PRPs,

respectively, and may respond differently to ProGln-
terminating peptides. Alternatively, both the N-terminal and
middle/C-terminal peptides could act as inhibitors or
retarders of plaque formation in a broader sense.

5

While the inventors believe these explanations to be true
they are of a hypothetical nature and should not be
understood so as to limit the scope of the invention which
is only determined by the appended claims.

10

According to the invention is disclosed an oligopeptide
protecting against dental caries comprising two arginine
residues selected from the group consisting of pentapeptide,
hexapeptide, heptapeptide, octapeptide, nonapeptide and
15   decapeptide. Particularly preferred is the pentapeptide
ArgGlyArgProGln.

Also preferred is a penta- to decapeptide comprised by the
sequence of amino acid 99 to amino acid 115 of the 150
20   residue PRP-1 protein:
GlyGlyHisProArgProProArgGlyArgProGlnGlyProProGlnGln,
with the provisio that it contains two or more Arg.

Also preferred are the following peptides:
25   ArgGlyArgProGln (residues 106-110);
ArgGlyArgProGlnGly (residues 106-111);
ArgGlyArgProGlnGlyPro (residues 106-112);
ArgGlyArgProGlnGlyProPro (residues 106-113);
ArgGlyArgProGlnGlyProProGln (residues 106-114);
30   ArgGlyArgProGlnGlyProProGlnGln (residues 106-115);
GlyGlyHisProArgProProArgGlyArg (residues 99-108);
GlyHisProArgProProArgGlyArg (residues 100-108);
HisProArgProProArgGlyArg (residues 101-108);
ProArgProProArgGlyArg (residues 102-108);

ArgProProArgGlyArg(residues 103-108);
ProProArgGlyArg (residues 104-108).

The peptides of the invention can be easily synthesized by
5   solid phase or liquid phase methods known in the art.

According to the invention is disclosed a method of
preventing dental caries comprising the oral administration
of a prevention-effective amount of an oligopeptide
10   comprising two arginine residues selected from the group
consisting of pentapeptide, hexapeptide, heptapeptide,
octapeptide, nonapeptide and decapeptide. Particularly
preferred is the pentapeptide ArgGlyArgProGln for use in the
method of preventing dental caries.

15
Also preferred for use in the method of preventing dental
caries is a penta- to decapeptide comprised by the sequence
of amino acid 99 to amino acid 115 of the 150 residue PRP-1
protein:GlyGlyHisProArgProProArgGlyArgProGlnGlyProProGlnGln,
20   with the provisio that it contains two or more Arg.

Also preferred for use of preventing dental caries are the
following peptides:
ArgGlyArgProGln (residues 106-110);
25   ArgGlyArgProGlnGly (residues 106-111);
ArgGlyArgProGlnGlyPro (residues 106-112);
ArgGlyArgProGlnGlyProPro (residues 106-113);
ArgGlyArgProGlnGlyProProGln (residues 106-114);
ArgGlyArgProGlnGlyProProGlnGln (residues 106-115);
30   GlyGlyHisProArgProProArgGlyArg (residues 99-108);
GlyHisProArgProProArgGlyArg (residues 100-108);
HisProArgProProArgGlyArg (residues 101-108);
ProArgProProArgGlyArg (residues 102-108);
ArgProProArgGlyArg(residues 103-108);

ProProArgGlyArg (residues 104-108).

According to the invention is disclosed a composition for
preventing dental caries comprising a prevention-effective
5   amount of an oligopeptide comprising two arginine residues
selected from the group consisting of pentapeptide,
hexapeptide, heptapeptide, octapeptide, nonapeptide and
decapeptide, and a suitable carrier. Particularly preferred
is the pentapeptide ArgGlyArgProGln. Suitable carriers
10   include state-of-the-art toothpaste and mouthwash
compositions but also chewing-gums, lozenges, and the like.

Also preferred for use in the composition for preventing
dental caries is a penta- to decapeptide comprised by the
15   sequence of amino acid 99 to amino acid 115 of the 150
residue PRP-1 protein:
GlyGlyHisProArgProProArgGlyArgProGlnGlyProProGlnGln,
with the provisio that it contains two or more Arg.

20   Also preferred for use in the composition for preventing
dental caries are the following peptides:
ArgGlyArgProGln (residues 106-110);
ArgGlyArgProGlnGly (residues 106-111);
ArgGlyArgProGlnGlyPro (residues 106-112);
25   ArgGlyArgProGlnGlyProPro (residues 106-113);
ArgGlyArgProGlnGlyProProGln (residues 106-114);
ArgGlyArgProGlnGlyProProGlnGln (residues 106-115);
GlyGlyHisProArgProProArgGlyArg (residues 99-108);
GlyHisProArgProProArgGlyArg (residues 100-108);
30   HisProArgProProArgGlyArg (residues 101-108);
ProArgProProArgGlyArg (residues 102-108);
ArgProProArgGlyArg (residues 103-108);
ProProArgGlyArg (residues 104-108).

Since the caries-protecting peptides according to the invention are peptides produced by the human body or correspond to sequences thereof they are well tolerated. Optimal caries-protecting amounts of these peptides can be
5   easily determined by experiment because the absence of important toxicity restraints. Single doses from 0.1 mg to 1 g will be appropriate for most applications.

The invention will be better understood by reference to a
10   drawing and the following description of preferred embodiments thereof.

DESCRIPTION OF THE DRAWING

15   Fig. 1   illustrates the native alkaline PAGE patterns of N-terminal acidic peptides from degradation of acidic PRPs (PRP-1 and PRP-3) by strains of commensal Streptococcus and *Actinomyces* species;

Fig. 2   illustrates the gel filtration patterns of
20   fragments formed by degradation of PRP-1 by strains representative for each of the four degradation patterns identified among streptococci and actinomycetes;

Fig. 3a   illustrates the gel filtration patterns of peptide
25   fragments from the degradation of PRP-1 by *S. gordonii* strain SK12 for different time periods;

Fig. 3b   illustrates the identification by mass spectrometry of fragments separated using either gel filtration (peaks 1-4, cf. Fig. 3a) or peptide gel filtration
30   (peak 5).

DESCRIPTION OF PREFERRED EMBODIMENTS

EXAMPLE 1. Isolation of acidic PRPs. Parotid saliva was
collected from three subjects homozygous for PRP-1 and PIF-s
5 using Lashley cups and mild acidic lozenge stimulation.
After pooling of the salivas and dilution (1:1 by volume)
with 50 mmol/L Tris-HCl, 25 mmol/L NaCl, pH 8.0 (Tris-HCl
buffer), the saliva sample was subjected to DEAE-Sephacel
column chromatography (15 x 1.6 cm, Pharmacia, Uppsala,
10 Sweden) using a linear gradient of 25 to 1000 mmol/L NaCl in
Tris-HCl buffer. The protein fraction containing the acidic
PRPs (PRP-1/PIF-s and PRP-3/PIF-f) was concentrated via
ultrafiltration using a Centriprep 10 concentrator (Amicon
Inc., Beverly, MA), and subjected to gel filtration (HiLoad™
15 26/60 Superdex S-200 Prep grade column, Pharmacia) in 20
mmol/L Tris-HCl, 500 mmol/L NaCl, pH 8.0. The resolved
protein fractions (PRP-1/PIF-s and PRP-3/PIF-f) were
dialyzed against Tris-HCl buffer (Spectra/Pore membrane No.
4, Spectrum Medical Industries, Inc., Houston, TX). Each
20 protein fraction (PRP-1/PIF-s and PRP-3/PIF-f) was finally
purified on a Macroprep high Q column (15 x 1.6 cm, Bio-Rad,
Hercules, CA) using a linear gradient of 25 to 1000 mmol/L
NaCl in 50 mmol/L Tris-HCl, pH 8.0. The purified proteins
were extensively dialyzed against water, lyophilized and
25 stored at -20°C.

EXAMPLE 2. Bacterial strains and culturing. The origins and
species designations of strains of *Actinomyces naeslundii*,
Actinomyces odontolyticus, *A. naeslundii genospecies* 1
30 strain ATCC 12104, genospecies 2 strain T14V, *A.*
odontolyticus strain PK984 and *A. viscosus* strain 19246 are
as previously described (Hallberg K et al, see above;
Hallberg K et al., Oral Microbiol Immunol 13 (1989) 188-192;
Li et al., 1999, see above). The strains of *S. anginosus, S.*

gordonii, S. mitis, S. oralis and *S. sanguis* (provided by
Dr. M. Kilian, Department of Oral Biology, Royal Dental
College, Aarhus, Denmark) have been described (Kilian et
al., 1989; Hsu et al., 1994). *S. mutans* strains Ingbritt,

5   JBP and NCTC 10449, *S. sobrinus* strain SL-1 and
Porphyromonas gingivalis strains W83 and ATCC 33277 were
provided by Dr. J. Carlsson, Department of Oral Biology,
Umeå University, Sweden. All strains were grown overnight on
Columbia-II-agar base plates (Becton Dickinson and Company,

10   Cockeysville, MD), supplemented with 30 ml of a human
erythrocyte suspension per liter, at 37°C in an atmosphere
with 5 % CO2. Growth of *S. gordonii* strain SK12 identified
two morphological types, one forming flat (strain SK12-I)
and the other convex (strain SK12-II) colonies. Strain SK12-

15   I (the flat colony-forming type) was selected for the
degradation experiments. Neither whole cell soluble protein
patterns, as analyzed by SDS-PAGE, nor acidic PRP cleavage
patterns distinguished between the two types. The strains
intended for degradation experiments were then grown at 37°C

20   for 18 h in 5 mL trypticase soy bean glucose limiting broth
(1.7% peptone, 0.3% soy peptone, 0.15% yeast extract, 12.5
mmol/L glucose, 12.5 mmol/L NH_4HCO_3 in 1 mol/L NaH_2PO_4/K_2HPO_4
buffer, pH 7.3) in an atmosphere with 5% CO_2. The cells were
pelleted by centrifugation at 17,000 x g for 5 min, washed

25   twice in 0.43% NaCl, 0.042% KCl, 0.1% Na_2HPO_4, 0.1% KH_2PO_4,
1% glycerophosphate disodium salt, 0.024% $CaCl_2$, and 0.01%
$MgCl_2.H_2O$ (M-DIL buffer), re-suspended in M-DIL buffer at a
concentration of 2 x 109 cells/mL. The bacterial cells (and
cell-free supernatant after pelleting of bacteria by

30   centrifugation) were kept on ice prior to degradation
experiments. The strains intended for adhesion tests were
metabolically labeled by adding 35S-methionine (200 *Ci,
Tran 35S-Label, ICN Pharmaceuticals Inc., Irvine, CA) to
bacteria suspended in 100 µL 10 mmol/L Na_2HPO_4, NaH_2PO_4,

0.137 M NaCl, pH 7.2, (PBS) prior to growth on Columbia-II-agar plates.

EXAMPLE 3. Degradation of acidic PRPs. Equal volumes (200 µL) of purified protein (0.6 mg/mL) and bacteria (2 x 109 cells/mL), both dissolved in M-DIL buffer, were mixed and incubated at 37°C for 15 min, 4 h, 20 h and 1 week. After pelleting of the bacteria by centrifugation at 17,000 x g for 10 min, the supernatants were aliquoted, lyophilized and stored at -80°C prior to analysis. In some experiments, cell free enzyme supernatants and parotid saliva from defined acidic PRP phenotypes were used following the same protocol. The saliva was collected as described above and sterilized by filtering (0.20 µm Minisart filter, Sartorius, Göttingen, Germany).

EXAMPLE 4. Native alkaline PAGE. Native alkaline polyacrylamide gel electrophoresis was performed essentially as described (Azen and Yu, 1984). The lyophilized supernatants were dissolved in 50 µL sample buffer containing 1% glycine, 10% glycerol and 0.025% bromophenol blue and centrifuged at 17,000 x g for 10 min. The electrophoresis was performed using precast Tris-glycine 7.5% resolving gels with 4% stacking gels (Bio-Rad) at 100 V for 20 min followed by 175 V for 1 h 30 min in a buffer containing 0.038 M Tris-glycine, pH 8.4. The gels were stained with 0.1% Coomassie Blue R (Serva Feinbiochemica, Heidelberg, Germany) in 20% trichloroacetic acid (Merck, Darmstadt, Germany) for 16 h, destained in 2% acetic acid for 16 h and finally soaked in water for 2 h.

EXAMPLE 5. Densitometry. The degree of acidic PRP degradation was quantified by densitometry of native alkaline PAGE gels using a Model GS-700 Imaging densitometer

and the Molecular Analyst Software (Bio-Rad). The degree of degradation of PRP-1 and PRP-3 was scored from 0 to 5 according to the following criteria: 0 = 0<10% , 1 = 10<20%, 2 = 20<40%, 3 = 40<60%, 4 = 60<80%, 5 = 80<100% reduction of

5 PRP-1 or PRP-3 at 4 h of incubation. In the case of certain strains, score 1 denotes the formation after 20 h of incubation of peptide fragments similar to those generated after 4 h by the strains with a high PRP degradation rate (Table 1).

10

EXAMPLE 6. Gel filtration. The lyophilized supernatants obtained by incubation of bacteria with acidic PRPs were dissolved in 200 µL 20 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl and subjected to gel filtration on Superose 12 HR 10/30

15 or a Superdex Peptide HR10/30 (Pharmacia) columns, equilibrated in 20 mM Tris-HCl, pH 8.0. The flow rate was 0.2 mL/min and the absorbance was monitored at 214 nm. Fractions of 1 mL were collected.

20 EXAMPLE 7. Hydroxyapatite assay. Adherence of 35S-methionine labeled bacteria to acidic PRP-1 was measured by the hydroxyapatite assay as previously described (Carlén et al., 1998).

25 EXAMPLE 8. Mass spectrometry. All mass spectrometric data were acquired on a hybrid quadrupole time-of-flight (Q-ToF) mass spectrometer (Micromass, Manchester, UK). A Z-configured nano spray source was used and samples were introduced via gold-coated spraying needles (Protana,

30 Odense, Denmark). Detection was all times in the positive ion mode. Needles were opened manually under a light-microscope resulting in an opening of approximately 5 µm. Before analysis, remaining contaminants and salt were removed by applying the samples to nano-columns; Gel-loader

tips (Eppendorf, Hamburg, Germany) packed with POROS R3
reverse phase resin (PerSeptive Biosystems, Framingham, MA,
USA) to a bed volume of approximately 200 nL. Normally, 5 µL
of sample was applied to the resin followed by washing with
5   approximately 30 µL 0.1% TFA and elution with 60% CH3CN in
1% acetic acid directly into the nano-spray needle.

EXAMPLE 9. N-terminal sequence analysis. Edman degradation
was performed directly on proteins blotted (Transblot, Bio-
10   Rad) onto PVDF-membranes using an Applied Biosystems 477A
pulsed liquid phase sequencer (Foster City, CA) with an on-
line PTH 120A analyzer. Sequence analysis was performed with
cycle programs adapted to the reaction cartridges and
chemicals from the manufacturer. Yields were calculated from
15   sequenced standard *-lactoglobulin.

EXAMPLE 10. Substrate specificity. The substrate specificity
of bacterial strains was measured as previously described
(Erlanger et al., 1961). Briefly, 25 µL of bacterial
20   suspension (5 x 109 cells/mL in M-DIL buffer) was diluted
with 25 µL of 0.1 mol/L Tris-HCl, pH 7.0, followed by
addition of 50 µL of B-Arg-pNA (Sigma, St. Louis, MO) and Z-
Gly-Pro-pNA (Bachem, Bulendorf, Switzerland) (8.0 mmol/L in
DMSO). After incubation at 37°C for 16 h, the extent of
25   cleavage was measured by the absorbance at 414 nm.

EXAMPLE 11. Degradation of acidic PRPs by commensal
Streptococcus and Actinomyces species. Strains of
Streptococcus and Actinomyces species were tested for
30   degradation of acidic PRPs (PRP-1 and PRP-3) by co-
incubation of bacteria and protein followed by native
alkaline PAGE (Table 1, Fig. 1). Degradation of PRP-1
occurred with S. oralis (1 of 4 strains), S. mitis (1 of 4
strains), S. gordonii (3 of 4 strains), S. sanguis (4 of 4

strains), *S. anginosus* (3 of 4 strains) and *A. odontolyticus* (4 of 7 strains), but not with *S. mutans* (3 strains), *S. sobrinus* (1 strain), *A. naeslundii* genospecies 1 (6 strains) and genospecies 2 (7 strains) or *A. viscosus* (1 strain). *S.*

5   *gordonii* and *A. odontolyticus* displayed high PRP-1 cleavage rates (scores 4 and 5), while other species had moderate (scores 2 and 3) to low (score 1) cleavage rates. While most species showed high PRP-1 relative to PRP-3 cleavage rates, *A. odontolyticus* displayed equal PRP-1 and PRP-3 cleavage

10   rates. Degradation of PRP-1 also occurred in cell-free supernatants (Table 1), and *S. gordonii* strain SK12 (with a high PRP-1 cleavage rate) degraded PRP-1, PRP-2, Db-s, PIF-s and Pa in parotid saliva from defined donors (data not shown).

15

Both streptococci adhering to and those not adhering to PRP-1 degraded acidic PRPs (Table 1). While *A. naeslundii* genospecies 1 and 2 bound to but did not degrade acidic PRPs, the opposite was true for *A. odontolyticus*. In

20   addition, both S-IgA1 protease positive and negative strains degraded acidic PRPs (Table 1). Thus, no obvious relationship was found between PRP degradation and adhesion to acidic PRPs or S-IgA1 protease activity.

25   EXAMPLE 12. Time-dependency of acidic PRP degradation. The native alkaline PAGE patterns from degradation of acidic PRPs for different times indicated a time-dependency of degradation for all strains (Fig. 1). The time-dependency of degradation of PRP-1 by *S. gordonii* strain SK12 was further

30   analyzed by gel filtration of fragments formed after different times of incubation (Fig. 3a). Degradation of PRP-1 (peak 1) for 15 min generated a peptide eluting similar to PRP-3 (peak 2) and later-eluting, smaller peptides (peak 3). Prolonged degradation (20 h) completely converted PRP-1 into

the peptide eluting similar to PRP-3 (peak 2) and even later-eluting peptides (peak 4). Even further degradation (1 week) completely transformed all peptides (except for the one eluting similar to PRP-3) into oligopeptides and amino

5   acids (peak 5), as identified using peptide gel filtration (data not shown).

EXAMPLE 13. Identity and structural features of acidic PRP-derived peptides. The peptides generated from PRP-1 by *S.*

10   *gordonii* strain SK12 were identified by mass spectrometry of the peptide peaks obtained by gel filtration (Figs 2 and 3). An N-terminal 105 residue peptide Pyr1-Pro104Pro105 (peak 2) and a C-terminal 40 residue peptide Gly111-Pro149Gln150 (peak 3) were identified after initial (15 min) cleavage of

15   PRP-1 (Fig. 3b). A series of 15-47 residue peptides (peak 4; Pro96-Pro109Gln110, Gly111-Pro130Gln131, Gly111-Pro134Pro135, Gly111-Pro135Gln136, Gly111-Pro140Gln141, Gly111-Pro149Gln150 and Pro104-Pro149Gln150) were identified after prolonged degradation (20 h). In addition, a

20   pyroglutamatic acid, Pyr, was found at the N-terminal residues of PRP-1 and PRP-3 (Fig. 3b), explaining our difficulties in sequencing of N-terminal fragments by Edman degradation.

25   EXAMPLE 14. Patterns of acidic PRP degradation. The peptide profiles displayed by native alkaline PAGE distinguished four major degradation patterns, I-IV (Table 1, Fig. 1). While all four patterns contained N-terminal peptides migrating close to PRP-3, the peptide banding patterns were

30   unique to a particular strain or species (Fig. 1, Table 1). Both species specific (*A. odontolyticus* and *S. gordonii*) and multiple patterns within a species (*S. anginosus* and *S. sanguis*), as well as a sharing of patterns between species, were observed. In addition, gel filtration of the

degradation products obtained by a strain representative of each pattern verified somewhat deviating profiles, containing large (70-75 min retention) and intermediate (80-95 min retention) peptides (Fig 1b). To investigate the

5 substrate specificities associated with acidic PRP degradation, strains representative of each degradation pattern were tested for truncation of Pro- and Arg-containing chromogenic substrates. The strains did not cleave Z-Gly-Pro-pNA, which is a substrate for prolyl

10 endoproteases (Blumberg et al., 1980), or B-Arg-pNA, while P. gingivalis strain ATCC 33277 cleaved both substrates.

EXAMPLE 15. Lozenge. A solution of ArgGlyArgProGln 'acetate' was prepared by dissolving ArgGlyArgProGln in

15 water and adding acetic acid to pH 6.5. The aqueous solution was freeze-dried and the powder thereby obtained mixed with 150 g of polyyethylene glycol 8000, 150 g of microcrystalline cellulose, 600 g of mannitol, 10 g of stearic acid are milled to pass a 40 mesh sieve. The mixture

20 is fed to a tablet press to produce 1 g tablets.

EXAMPLE 16. Chewable tablet. 900 g mannitol and 5 g sodium saccharin are screened through a 40-mesh screen and blended thoroughly with 40 g ArgGlyArgProGln acetate prepared as

25 described above. A binder solution of 20 g of acacia and 50 g of gelatin in 500 ml water was prepared separately. The powder was wet granulated using 200 ml of binder solution for 1000 powder. After drying overnight at 75°C the granules were screened through a 12 mesh screen, mixed with 1 g of

30 peppermint oil adsorbed on 3 g of colloidal silica and 25 g magnesium stearate. From this mixture 1 g tablets were compressed to a hardness of 12 kg.

EXAMPLE 17. Toothpaste. A suitable toothpaste base for incorporating the compounds of the invention is described in U.S. Patent No. 3,935,305 (Delaney et al.) which is incorporated herein by reference; in particular, see Example

5   1. A suitable amount of ArgGlyArgProGln acetate (for preparation, see above) is 1% by weight of base.

EXAMPLE 18. Mouthwash. A suitable mouthwash base for incorporating the compounds of the invention is described in

10   U.S. Patent No. 5,145,664 (Thompson) which is incorporated herein by reference; see Example 1. A suitable amount of ArgGlyArgProGln acetate (for preparation, see above) is 1% by weight of base.

15   LEGENDS TO FIGURES

Fig 1. Native alkaline PAGE patterns of N-terminal acidic peptides from degradation of acidic PRPs (PRP-1 and PRP-3) by strains of commensal Streptococcus and *Actinomyces*

20   species. Shown are the degradation patterns of strains representative for each of the four degradation patterns (I-IV) identified among isolates of streptococci and actinomycetes (cf. Table 1). All strains (Table 1) were co-incubated with purified PRP-1 and PRP-3 for different times

25   (15 min, 4h and 20 h) and analyzed for such peptide patterns.

Fig. 2. Gel filtration patterns of fragments formed by degradation of PRP-1 by strains representative for each of

30   the four degradation patterns identified among streptococci and actinomycetes (cf. Table 1). The strains were incubated with PRP-1 for 15 min and 20 h. The retention times of purified PRP-1 and PRP-3 are indicated by vertical arrows.

Fig 3a. Gel filtration patterns of peptide fragments from degradation of PRP-1 by *S. gordonii* strain SK12 for different times. The numbering of the peaks refer to mass spectrometric identification of the corresponding peptide

5   structures (cf. Fig. 3b). The vertical arrows denote the retention times of purified PRP-1 and PRP-3.

Fig. 3b. Identification by mass spectrometry of fragments separated using either gel filtration (peaks 1-4, cf. Fig.

10   3a) or peptide gel filtration (peak 5). The numbering of peptides by letters A-G denotes the corresponding signals in the mass spectrum. Pyr indicates a pyroglutamic acid. The mass numbers with an asterisk indicate average masses from de-convoluted mass spectra, while unlabeled mass numbers are

15   consistent with the monoisotopic mass of the peptide.

20

25

30

Table 1. Degradation of acidic PRPs by *Streptococcus* and *Actinomyces* species

Species[a]	Strain[a]	Acidic PRP degradation[b]			PRP-1 adhesion[d]	IgA1 protease[e]
		PRP-1	PRP-3	Type[c]		
S. gordonii	SK 12, SK 184	5	1	I	-	-
	SK 120	5	0	I	+	-
	SK 33	0	0	-	-	-
S. sanguis	SK 85	4	0	I	-	+
	SK 112	2	1	II	+	+
	SK 37	2	1	II	-	+
	SK 162	2	1	III	-	+
S. mitis	SK 304	1	0	II	-	-
	SK 305	0	0	-	+	-
	SK 96, SK 142	0	0	-	-	-
S. anginosus	SK 215	3	1	III	-	-
	SK 52	1	1	II	-	-
	SK 63	1	0	II	-	-
	SK 218	0	0	-	-	-
S. oralis	SK 143	1	0	IV	-	+
	SK 2, SK 92	0	0	-	-	+
	C 104	0	0	-	nt	+
S. mutans	Ingbritt, JBP	0	0	-	-	nt
	NCTC 10449	0	0	-	nt	nt
S. sobrinus	SL-1	0	0	-	nt	nt
A. odontolyticus	T-5-G	5	5	IV	+	nt
	T-1-K	2	1	IV	-	nt
	T-23-N,T-3-G	1[b]	1	IV	-	nt
	T-21-N, T-22-N, PK 984	0	0	-	-	nt
A. naeslundii genospecies 1	ATCC 12104, P-3-N, P-5-N, P-11-N, B-2-G, PK 947	0	0	-	-	nt
A. naeslundii genospecies 2	T14V, P-1-N, P-7-N, P-1-K, P-1-G, B-7-N, P-2-N	0	0	-	+	nt
A. viscosus	19246	0	0	-	-	nt
P. gingivalis	W83	5	5	-	-	+

a) The sources of the strains are given above.

b) Acidic PRP degradation was carried out by co-incubation of PRP-1 or PRP-3 and bacteria for 15 min, 4 h and 20 h followed by native alkaline PAGE (Fig.1). The degree of cleavage at 4 h was scored from 0 to 5 by densitometry: $0 = 0<10$ %, $1 = 10<20$ %, $2 = 20<40$ %, $3 = 40<60$ %, $4 = 60<80$ %, and $5 = 80<100$ % loss of acidic PRPs. Score 1 labeled with a superscript denotes the formation of degradation products. Cells free supernatant resulted in similar degradation

patterns for most strains. 95 % confidence intervals of densitometric runs corresponded to 8 % of mean.

c) The strains were classified into degradation patterns I-IV based on native alkaline PAGE peptide profiles (Fig.1). The type-I pattern displayed a peptide migrating just below PRP-3; The type-II pattern a peptide migrating just below PRP-3 and another between PRP-1 and PRP-3; The type-III pattern two peptides migrating just below and above PRP-3, one between PRP-1 and PRP-3 and a fourth just below PRP-1; and the type-IV pattern a peptide migrating identical to PRP-3 and another between PRP-1 and PRP-3 although closer to PRP-1 compared to patterns II and III.

d) Adhesion of ^{35}S-labeled bacteria to PRP-1-coated hydroxyapatite beads. Adhesion exceeding 15 % of added cells was considered positive. Adhesion of *Actinomyces* was tested at 5µg/ml of PRP-1 and for other bacteria at 30 µg/ml of PRP-1. The criteria for positive binding and assay conditions differ from those in other studies of adhesion of streptococci and *P. gingivalis* (Hsu et al., 1994).

e) S-IgA protease activity of commensal streptococci (Kilian et al., 1989), *P. gingivalis* (Kilian, 1981) and *Actinomyces* species, which lack S-IgA1 protease activity (M. Kilian, personal communication).

Table 2. Simplified map of generated peptides in comparison
with PRP-1.

Peak	Peptide		Mass (Da)
1	Pyr1----Pro149-Gln150		15 514.0*
2	Pyr1----Pro104-Pro105		11 005.5*
3	Gly111---Pro149-Gln150		3 930.0
4	Pro96----Pro109-Gln110	(A)	1 663.7
4	Gly111---Pro130-Gln131	(B)	2 082.1
4	Gly111---Pro134-Pro135	(C)	2 430.4
4	Gly111---Pro135-Gln136	(D)	2 558.4
4	Gly111---Pro140-Gln141	(E)	3 053.6
4	Gly111---Pro149-Gln150	(F)	3 930.0
4	Pro104---Pro149-Gln150	(G)	4 718.3
5	Oligopeptides/amino acids		

The potential cleavage sites at peptide bonds formed at Pro or
Gln residues are given. The presumed Arg-containing pentapeptide
is blown up, ProGln-termini are marked by black circles and the
post-translational cyclization of the N-terminal Glu residue to a
pyroglutamic acid residue is marked Pyr. The verification by mass
spectrometry of phosphorylation of Ser at positions 8 and 22 is
marked.

24

Claims

1. An oligopeptide protecting against dental caries comprising two arginine residues selected from the group consisting of pentapeptide, hexapeptide, heptapeptide, octapeptide, nonapeptide and decapeptide.

2. The oligopeptide of claim 1 comprised by the sequence GlyGlyHisProArgProProArgGlyArgProGlnGlyProProGlnGln.

3. The oligopeptide of claim 2 ArgGlyArgProGln.

4. The oligopeptide of claim 2 selected from:

ArgGlyArgProGln;

ArgGlyArgProGlnGly;

ArgGlyArgProGlnGlyPro;

ArgGlyArgProGlnGlyProPro;

ArgGlyArgProGlnGlyProProGln;

ArgGlyArgProGlnGlyProProGlnGln;

GlyGlyHisProArgProProArgGlyArg;

GlyHisProArgProProArgGlyArg;

HisProArgProProArgGlyArg;

ProArgProProArgGlyArg;

ArgProProArgGlyArg;

ProProArgGlyArg.

5. A method of preventing dental caries comprising the oral administration of a prevention-effective amount of the peptide of any of claims 1-4.

6. A composition for preventing dental caries comprising a prevention-effective amount of the oligopeptide of any of claims 1-4 and a suitable carrier.
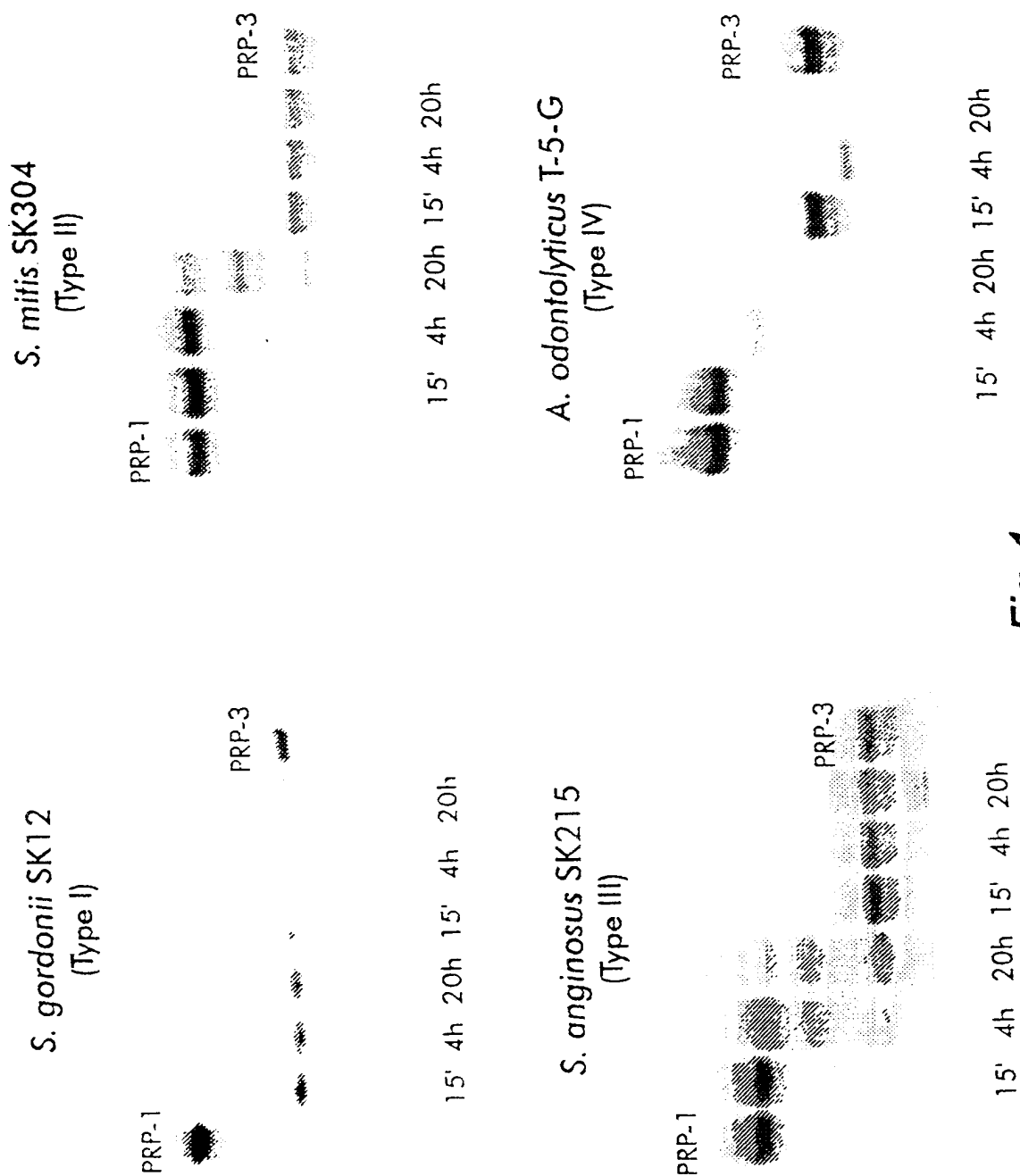
Fig. 1

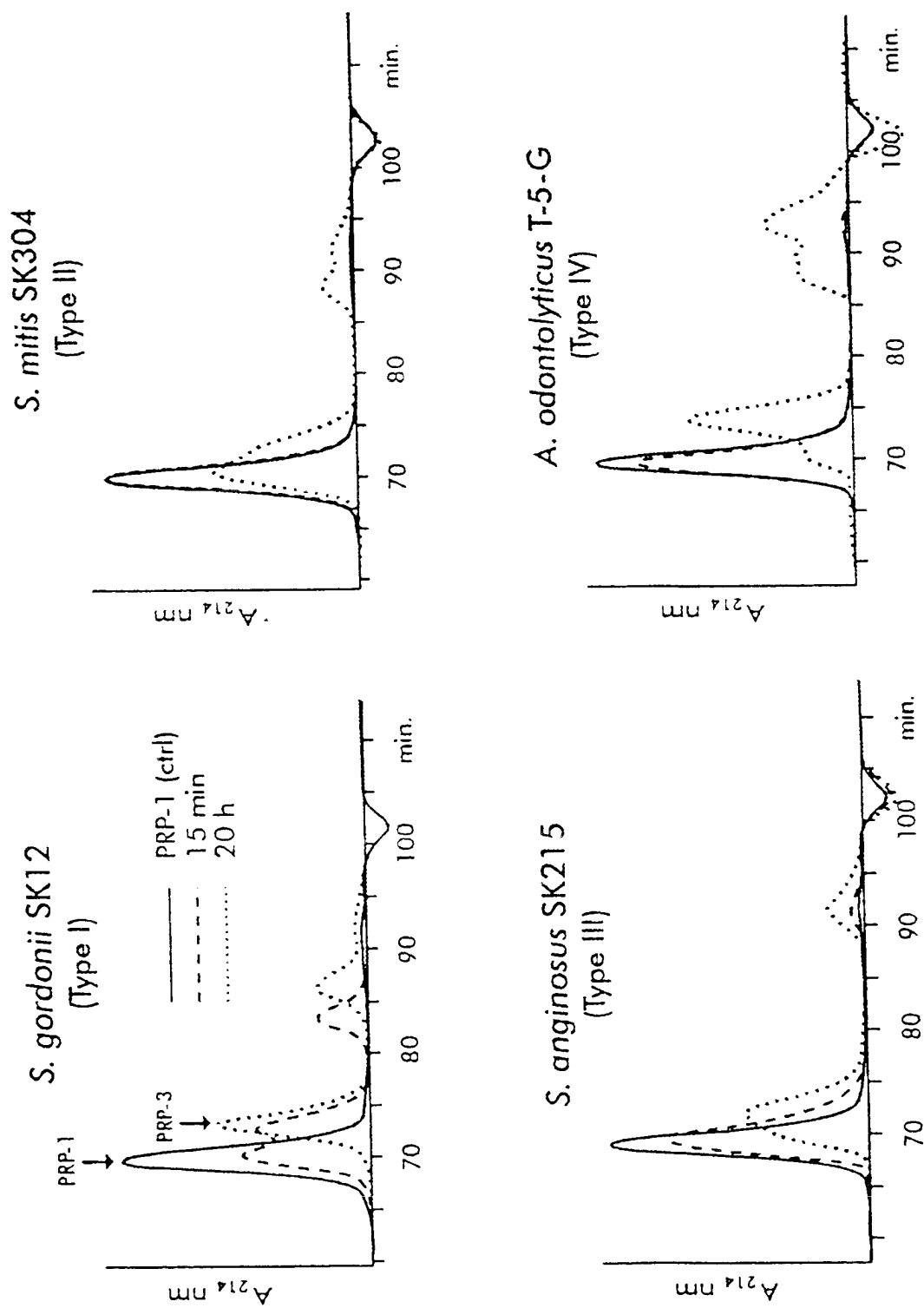Fig. 2

Fig. 3a

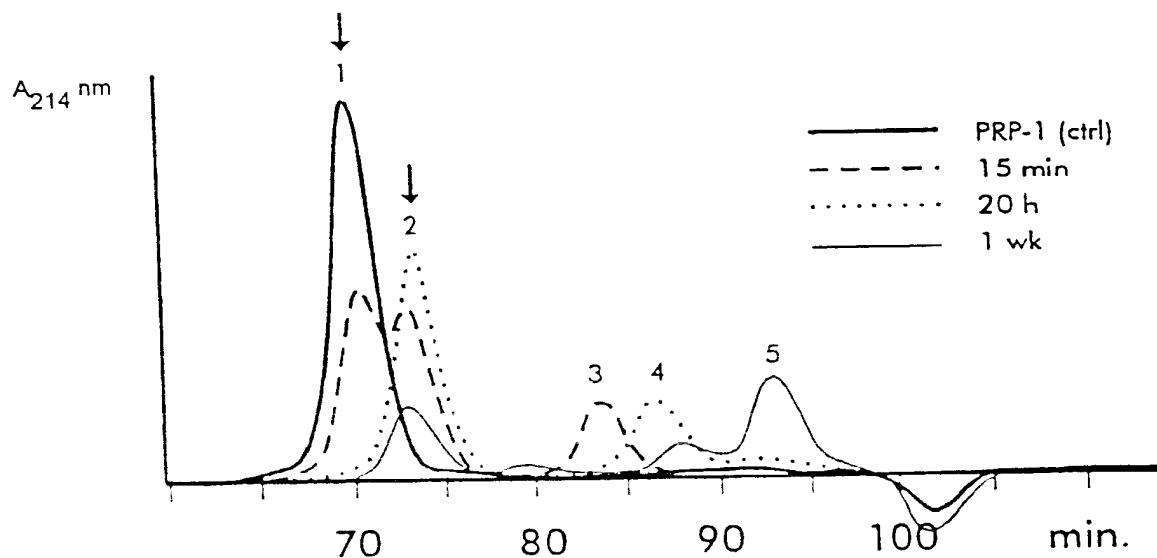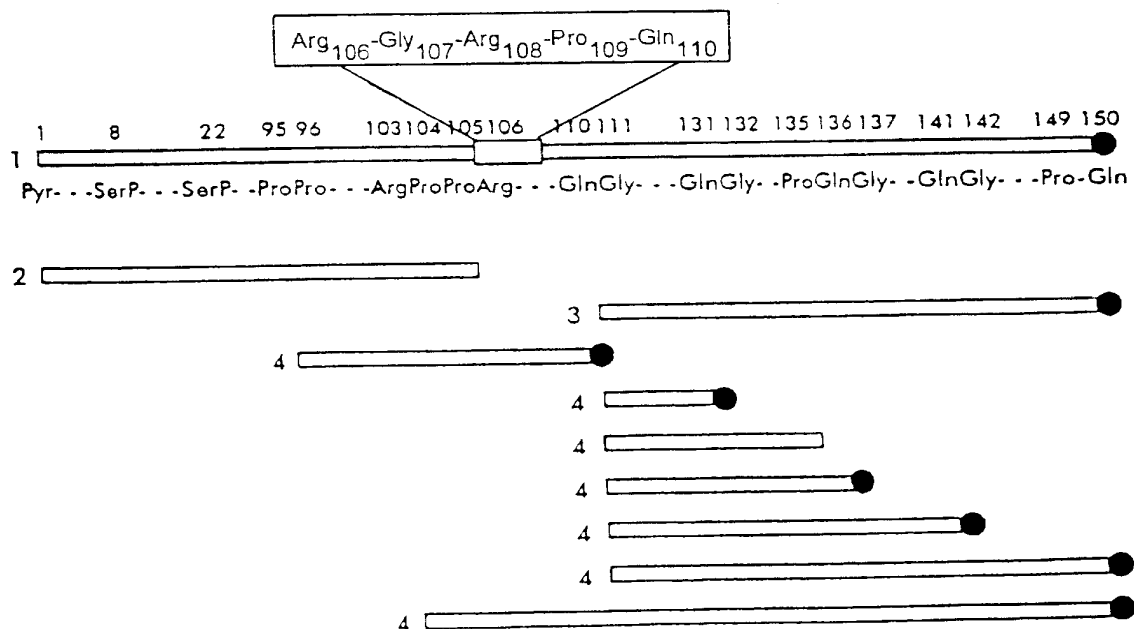Fig. 3b

SEQUENCE LISTING

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   Johansson, Ingegerd

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Gln

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07K 7/06, A61K 38/08, A61P 1/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4225579 A (ISRAEL KLEINBERG), 30 Sept 1980 (30.09.80)	1-8
X	WO 9914231 A2 (FORSSMANN, WOLF-GEORG), 25 March 1999 (25.03.99)	1-8
X	WO 9407523 A1 (EMORY UNIVERSITY ET AL), 14 April 1994 (14.04.94)	1-8
A	Journal of Immunology, Volume 152, 1994, Yasuo Yamauchi et al, "Recombinant and Native Zymogen Forms of Human Complement Factor D1" page 3645 - page 3652	4

☐ Further documents are listed in the continuation of Box C.   ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 Sept 2000	29 -09- 2000
Name and mailing address of the ISA Swedish Patent Office Box 5055, S-102 42 STOCKHOLM	Authorized officer Carolina Gómez Lagerlöf/GH

INTERNATIONAL SEARCH REPORT

Box I	Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international-type search report has not been established in respect of certain claims for the following reasons:

1. ☒ Claims No.: 5
   because they relate to subject matter not required to be searched by this Authority, namely:

   Claim 5 relates to a method of treatment of the human or animal body by surgery or by therapy/a diagnostic method practised on the human or animal body/ Rule. 39.1.(iv). Nevertheless, a search has been executed for this claim. The search has been based on the alleged effects of the compound/composition.

2. ☐ Claims No.:
   because they relate to parts of the national application that do not comply with the prescribed requirements
   to such an extent that no meaningful international-type search can be carried out, specifically:

Box II	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international-type search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international-type search report covers only those claims for which fees were paid, specifically claims No.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international-type search report is restricted to the invention first mentioned in the claims, it is covered by claims No.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Patent document cited in search report			Publication date	Patent family member(s)		Publication date
US	4225579	A	30/09/80	NONE		
WO	9914231	A2	25/03/99	DE	19805385 C	12/08/99
WO	9407523	A1	14/04/94	CA	2145313 A	14/04/94
				EP	0665754 A	09/08/95
				JP	8502262 T	12/03/96
				US	5447914 A	05/09/95
				US	5798336 A	25/08/98